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Expression of Double-Stranded-RNA-Specific RNase III of *Escherichia coli* Is Lethal to *Saccharomyces cerevisiae*

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The gene for the double-stranded RNA (dsRNA)-specific RNase III of *Escherichia coli* was expressed in *Saccharomyces cerevisiae* to examine the effects of this RNase activity on the yeast. Induction of the RNase III gene was found to cause abnormal cell morphology and cell death. Whereas double-stranded killer RNA is degraded by RNase III in vitro, killer RNA, rRNA, and some mRNAs were found to be stable in vivo after induction of RNase III. Variants selected for resistance to RNase III induction were isolated at a frequency of 4×10^{-5} to 5×10^{-5}. Ten percent of these resistant strains had concomitantly lost the capacity to produce killer toxin and M dsRNA while retaining L dsRNA. The genetic alteration leading to RNase resistance was localized within the RNase III-coding region but not in the yeast chromosome. These results indicate that *S. cerevisiae* contains some essential RNA which is susceptible to *E. coli* RNase III.

It is known that double-stranded RNA (dsRNA) plays various important roles in procaryotes, as well as in eucaryotes. For example, formation of dsRNA between mRNA and targeted antisense RNA blocks expression of a specific gene (for a review, see reference 8); formation of dsRNA is an important step during virus infection (e.g., see reference 24), RNA maturation (19), and interferon induction (14). *Escherichia coli* is known to contain a dsRNA-specific RNase called RNase III. This enzyme introduces specific cleavages in a number of RNA (rRNA and mRNA) precursors in *E. coli* as part of the RNA maturation process (6). In contrast to various single-stranded RNases, RNase III nonspecifically degrades dsRNA (21). RNase III is able to cleave RNA-RNA duplexes greater than 20 base pairs long, producing fragments an average size of 15 base pairs long (20). The *E. coli* RNase III gene has been cloned (25), and its DNA sequence has been determined (16, 18). It is intriguing to examine whether RNase III expression in eucaryotes results in specific effects on cell growth, gene regulation, cell physiology, cell morphology, or cell viability.

In this study, we examined expression of the *E. coli* RNase III gene in *Saccharomyces cerevisiae* and its effects on cell growth and cell RNA. We found that induction of expression of the RNase III gene resulted in abnormal cell morphology and cell death. We were able to isolate cells that became resistant to RNase III induction at a frequency of 4×10^{-5} to 5×10^{-5}. Among those resistant strains, approximately 10% were found to be cured of the killer factor M dsRNA. Analysis of the resistant strains revealed that the mutation was localized within the RNase III-coding region. These results indicate that yeast cells possess essential RNAs containing a double-stranded structure(s). Degradation of this double-stranded structure is considered to result in cell death.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The strain DMM1-15A (*leu2 ura3 ade2 his5*) and the plasmid YEp51 (2) were kindly provided by J. Broach of Princeton University, New Jersey. Strain S228 and the assay for yeast killer toxin were described by Sherman et al. (22). The growth medium (SD) used in all the experiments contained 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco Laboratories, Detroit, Mich.) and 2% (wt/vol) glucose, galactose, or raffinose and was supplemented with the appropriate amino acids. Cultures for growth experiments, RNA preparations, and labeling experiments were pregrown to the stationary phase (12 to 24 h) in SD-glucose medium at 30°C. These cultures were then inoculated into fresh SD medium supplemented with the appropriate carbon source. In growth experiments, culture turbidity was monitored by measuring optical density at 660 nm, and cell viability was monitored by plating appropriate dilutions on SD-glucose plates (2% agar). Lithium acetate DNA-mediated transformation was used for *S. cerevisiae* (11). *E. coli* SB221 and standard cloning techniques have been previously described (15). Variant strains resistant to RNase III induction were isolated by plating culture of DMM1-15A::pPY2 on SD-galactose plates.

Cell labeling and immunoprecipitation. *S. cerevisiae* DMM1-15A cells containing the appropriate plasmid were grown to 2×10^7 cells per ml, harvested, and resuspended in the same SD medium lacking methionine. After the cells were grown for 30 min at 30°C, 100 μCi of [^{35}S]methionine was added to a 5-ml culture and the cells were incubated for 1 to 2 h. The labeled cells were harvested, washed once with fresh SD medium, and suspended in 300 μl of TE (10 mM Tris, pH 8, 1 mM EDTA). Cells were broken with glass beads and centrifuged to recover a clear supernatant. This extract was then immunoprecipitated by RNase III antiserum and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as previously described (12).

Southern blot analysis. Yeast DNA was prepared as described by Sherman et al. (22). DNA was transferred to nitrocellulose essentially as described by Southern (23). An 850-base-pair fragment containing the RNase III gene was nick translated and used as the probe. Hybridization was performed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M

sodium citrate)–3× Denhardt solution (4)–0.5% SDS at 65°C for 12 to 16 h. The filters were washed in 3× SSC–0.5% SDS at 65°C for 2 h, dried, and analyzed by autoradiography.

Total yeast RNA isolation and RNA blot analysis. For preparation of total RNA from *S. cerevisiae*, 200-ml log-phase cultures (2×10^7 cells per ml) were harvested and suspended in 2.5 ml of LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris hydrochloride [pH 7.4], 0.2% SDS). Cells were then broken with glass beads in 3 ml of phenol equilibrated with LETS buffer. LETS buffer (5 ml) was added, and the aqueous phase was extracted twice with 5 ml of phenol-chloroform-isoamyl alcohol (25:25:1) and once with chloroform. Finally, total yeast RNA was precipitated with 0.1 volume of 5 M LiCl and 2 volumes of ethanol.

Nick translation of DNA probes and transfer of total RNA were performed essentially as described by Maniatis et al. (15). Filters were prehybridized at 42°C for 2 to 4 h in 5× SSC–50% formamide–5× Denhardt solution–0.5 mg of calf thymus DNA per ml–0.1% SDS. Hybridization was performed under the same conditions, except that the concentration of calf thymus DNA was 0.1 mg/ml and a denatured probe (2×10^7 to 3×10^7 cpm) was included. After hybridization for 16 to 20 h, filters were washed with a solution containing 50% formamide and 5× SSC at 42°C and then washed four times at 42°C with a solution containing 5 mM Tris hydrochloride (pH 7.0), 25 mM NaCl, 1 mM EDTA, and 0.1% SDS. The dried filters were then autoradiographed.

Radioactively labeled RNA was prepared by adding 1 mCi of $^{32}PO_4$ to cultures at 8 h after induction. The cells were labeled for 5 h and then extracted as described above. The amount of label in L dsRNA, 25S rRNA, and 18S RNA was determined in appropriate gel slices from a 0.5% agarose gel.

Assays for RNase III activity. The standard mixture (50 μl) for assaying RNase III contained 0.13 M NH_4Cl, 0.01 M magnesium acetate, 5% sucrose, and 0.02 M Tris hydrochloride (pH 7.9) as described by Robertson et al. (21). The [^{3}H] poly(A · U) copolymer substrate (21) was kindly provided by H. Robertson of Rockefeller University, and the reaction was performed at 37°C for 30 min and terminated by addition of 5% trichloroacetic acid. Bovine serum albumin carrier (0.2 mg) was added, and the precipitate was collected on Whatman GF/A glass fiber filter pads. The pads were dried and counted. An alternative substrate used a total yeast RNA preparation, described above, which was subjected to electrophoresis on a 0.5% agarose gel buffered with 40 mM Tris (pH 7.8)–20 mM sodium acetate–2 mM EDTA. RNase III activity was detected by specific degradation of L dsRNA at various RNase III concentrations (see Fig. 4). Purified RNase III was provided by J. Ahnn (unpublished data).

DNase I and RNase A hydrolysis. Total RNA preparations were incubated at 37°C for 30 min with DNase I (40 μg/ml) and RNase A (4 μg/ml) in a buffer containing 20 mM K_2HPO_4 (pH 7.6), 4 mM $MgCl_2$, and 0.6 M NaCl. To detect both L and M dsRNA species, total RNA samples were applied to a 3.5% polyacrylamide gel after DNase I and RNase A digestion. Electrophoresis was performed with 40 mM Tris hydrochloride (pH 7.8)–20 mM sodium acetate–2 mM EDTA.

RESULTS AND DISCUSSION

Expression of RNase III. To express RNase III in *S. cerevisiae*, a GAL10 expression system (2) was used which allows strong and rapid induction of transcription of the cloned gene when galactose is provided as a carbon source.

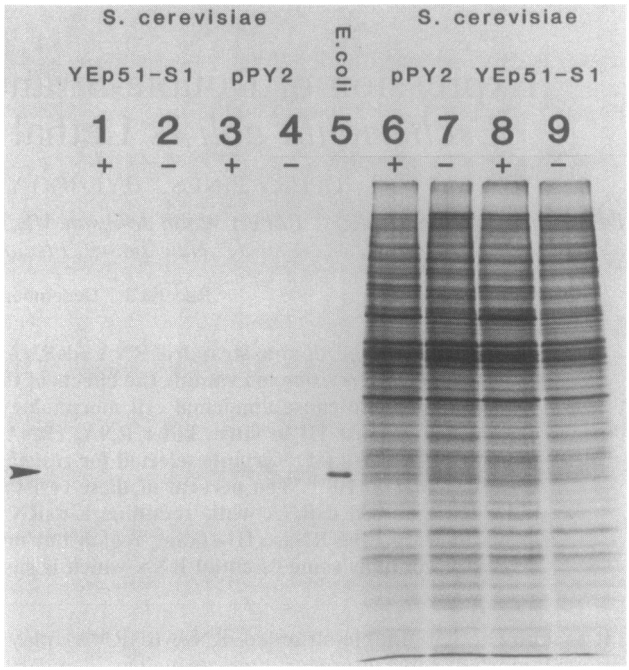

FIG. 1. Production of RNase III in *S. cerevisiae*. *S. cerevisiae* DMM1-15A cells harboring plasmid pPY2 or YEp51-S1 were grown to 10^7 cells per ml with raffinose (uninduced cultures) or galactose (induced cultures) as the carbon source and labeled for 1 h with [^{35}S]methionine. Samples of extracts prepared from the labeled cells were immunoprecipitated with antiserum to RNase III and then analyzed by SDS-polyacrylamide (17.5%) gel electrophoresis. Immunoprecipitated (lanes 1 to 4) and total (lanes 6 to 9) extracts of induced (+) and uninduced (−) cultures harboring either pPY2 (lanes 3, 4, 6, and 7) or YEp51-S1 (lanes 1, 2, 8, and 9) are presented. Lane 5 contained an immunoprecipitated extract of *E. coli* containing ^{35}S-labeled RNase III. The arrow shows the position of RNase III.

The shuttle vector YEp51 contains, in addition to the GAL10 promoter, the 2μm high-copy-number origin of replication and the yeast *LEU2* gene. An 850-base-pair *Hin*cII-*Bam*HI fragment from pTD101 (3) containing the RNase III gene was inserted into the *Sal*I and *Bam*HI sites of YEp51, thus placing the protein-coding sequence between the appropriate promoter and termination sites. The cloned fragment contains 24 base pairs upstream of the initiating ATG from the *E. coli* gene, in which there are no other initiation or termination codons.

To examine the expression of *E. coli* RNase III in *S. cerevisiae*, cells carrying YEp51-RNase III (designated pPY2) were labeled with [^{35}S]methionine and the total cell protein was analyzed by SDS-polyacrylamide gel electrophoresis. As a control throughout this study, the same strain (DMM1-15A) carrying a YEp51 derivative carrying the gene for protein S of *Myxococcus xanthus* (10) was used. A band with a mobility identical to that of *E. coli* RNase III was found in the extract from induced cultures harboring pPY2 (Fig. 1, lanes 6 and 7). A corresponding band was absent from extracts of cultures harboring YEp51-S1 which were induced under similar conditions (Fig. 1, lane 8). Immunoprecipitation of the above-described extracts with RNase III antiserum demonstrated that the suspected band was retained and thus contained material that cross-reacts with RNase III (compare lanes 3 and 4 in Fig. 1).

Strains containing either pPY2 or YEp51-S1 exhibited the

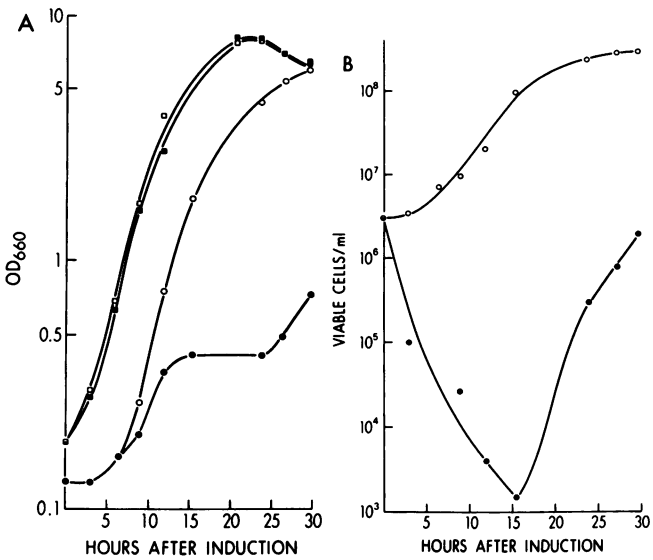

FIG. 2. Growth of *S. cerevisiae* DMM1-15A after induction of RNase III. DMM1-15A harboring pPY2 or YEp51-S1 was first grown to stationary phase in glucose-containing medium and then inoculated into either galactose- or glucose-containing medium. (A) Culture turbidity was monitored by optical density at 660 nm. (B) Cell growth was monitored by a viable count on glucose-containing plates. Symbols of respective plasmid and growth medium: ●, pPY2-galactose; □, pPY2-glucose; ○, YEp51-S1–galactose; ■, YEp51-S1–glucose.

same growth kinetics as monitored by culture turbidity when glucose was used as the carbon source (Fig. 2A). In contrast, when galactose was used as the carbon source, the strain harboring pPY2 grew more slowly than the strain harboring YEp51-S1 (Fig. 2A). The RNase III-induced culture stopped growing after 16 to 18 h at only about 20% of the turbidity of the control culture. The turbidity of the RNase III-induced culture eventually started to increase again because of the growth of a fraction of the culture which survived the lethal effect of RNase III induction. A striking effect on cell viability was observed after induction of RNase III (Fig. 2B). Within 3 h, the viability of induced cultures harboring pPY2 dropped 10-fold, and after 12 to 15 h the frequency of surviving cells was 4×10^{-5} to 5×10^{-5}. It was of interest that in the initial period after RNase III induction there was no change in cell morphology and the cells appeared to bud normally, as observed by phase-contrast light microscopy. However, after 10 to 12 h, the RNase III-induced cells appeared slightly larger than the control, and at 18 to 20 h, extremely large cells were observed (Fig. 3B), in contrast to control cells harboring YEp51-S1 (Fig. 3A). A yeast strain harboring one copy of the RNase III gene integrated into the chromosome displayed the same lethal effect and cell morphology in response to induction as the 2μm-derived pPY2 plasmid described (data not shown).

Activity of RNase III. Two assays were used to determine RNase III activity in yeast lysates. Initially we used the method of Robertson et al. (21), in which a ^{3}H-labeled double-stranded poly(A · U) copolymer is the RNase III substrate. Significant reduction in ^{3}H-labeled trichloroacetic acid-precipitable counts was found only in the lysate of the galactose-induced strain harboring pPY2 (data not shown). A second activity assay used naturally existing dsRNA from *S. cerevisiae*. Many *S. cerevisiae* strains contain dsRNA which is encapsidated in viruslike particles (27). Two types

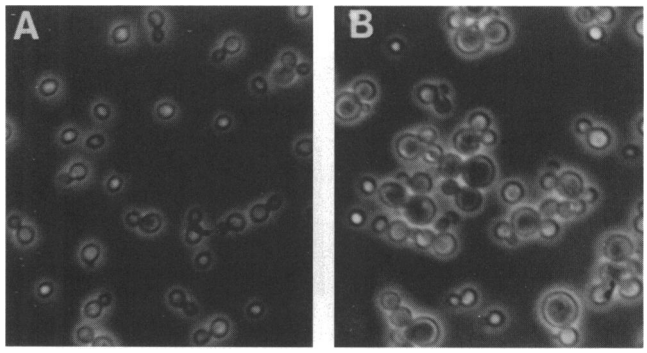

FIG. 3. Cell morphology after induction of RNase III. Cultures induced for 18 h (described in the legend to Fig. 2) harboring YEp51-S1 (A) or pPY2 (B) were subjected to phase-contrast light microscopy (magnification, ×640).

of dsRNA molecule are usually found in viruslike particles of *S. cerevisiae*. The larger, designated L, encodes the capsid protein (9), whereas the smaller dsRNA, designated M, encodes a toxin (1) which is secreted and can kill other *S. cerevisiae* strains. L dsRNA from a preparation of total RNA of *S. cerevisiae* was degraded by purified RNase III (Fig. 4). The dose-dependent degradation of L dsRNA appears to be specific, since rRNA (25S and 18S) was unaffected. Figure 4B presents the effects of crude yeast lysates on L dsRNA. Only lysates from cultures harboring pPY2 which were induced by galactose caused specific, dose-dependent digestion of L dsRNA (lanes 5 and 6). Lysates from uninduced cultures harboring pPY2 (lane 4) or lysates from strains harboring the control plasmid YEp51-S1 (lanes 2 and 3) exhibited no RNase III activity by this assay.

The lethality of RNase III induction in *S. cerevisiae* raises the question of the target of this enzyme in vivo. One possibility is that RNase III causes a general disruption of

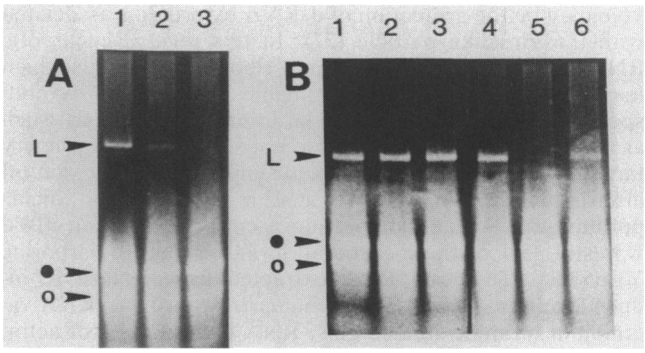

FIG. 4. Assay of RNase III activity in yeast lysates. (A) Total yeast RNA (20 μg) was treated with 0, 0.4, or 10 μg (lanes 1, 2, and 3, respectively) of purified RNase III at 37°C for 30 min, subjected to 0.5% agarose gel electrophoresis, and stained with ethidium bromide. (B) Strain DMM1-15A harboring pPY2 or YEp51-S1 was grown in either galactose- or glucose-containing medium as described in the legend to Fig. 2. Cultures (5 ml) were harvested at an optical density of 1 at 660 nm, and cell extracts were prepared. Samples of these extracts were incubated with total yeast RNA and subjected to gel electrophoresis as described above. The gel order in panel B with respect to the culture extract used (plasmid harbored-growth medium) was as follows: 1, no extract added; 2, 5 μl of YEp51-S1–glucose; 3, 5 μg of YEp51-S1–galactose; 4, 5 μl of pPY2-glucose; 5, 5 μl of pPY2-galactose; 6, 1 μl of pPY2-galactose. Symbols: L, L dsRNA; ●, 25S rRNA; ○, 18S rRNA.

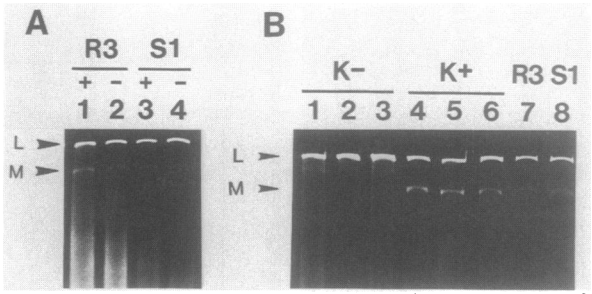

FIG. 5. (A) Stability of L and M dsRNAs in *S. cerevisiae* after induction. Total cell RNAs from cultures harboring pPY2 or YEp51-S1 grown for 6 h in either galactose (+)- or glucose (−)-containing medium were treated with RNase A and DNase I (as described in Materials and Methods), subjected to 3.5% polyacrylamide gel electrophoresis, and stained with ethidium bromide. Lanes: 1 and 2, RNA from DMM1-15A::pPY2; 3 and 4, RNA from DMM1-15A::YEp51-S1. (B) L and M dsRNAs in variants resistant to RNase III induction. Total cell RNAs from variants resistant to RNase III induction were analyzed as described for panel A. RNA was prepared from induced cultures of toxin-negative variants (K^-; lanes 1 to 3), toxin-positive variants (K^+; lanes 4 to 6), DMM1-15A::pPY2 (R3; lane 7), and DMM1-15A::YEp51-S1 (S1; lane 8).

RNA stability or synthesis or both. The double-stranded killer RNA was considered to be useful for probing RNase III in vivo since it is an RNase III substrate in vitro (Fig. 4). In contrast to the in vitro result, however, killer dsRNA appears to be stable after induction of RNase III in vivo. The polyacrylamide gel pattern for both L and M dsRNAs was the same with all four culture extracts examined (Fig. 5A). Furthermore, RNA preparations from cells that were labeled 8 h after induction incorporated the same proportion of $^{32}PO_4$ into L killer RNA relative to its incorporation into rRNA (data not shown). According to these results, RNase III does not cause specific degradation of L RNA in vivo, and we could not detect any effect on newly synthesized double-stranded L RNA.

A reasonable explanation for the stability of killer RNA in vivo may be the protection of dsRNA by proteins associated with the viruslike particle (27). In this regard, eucaryotic RNAs exist in vivo in the form of ribonucleoproteins which may render these RNAs unavailable for digestion by nonspecific RNases. RNAs involved in protein synthesis, such as rRNA, mRNA, and tRNA, do not seem to be the primary target of RNase III, since protein synthesis was not shut off after induction. Furthermore, at 12 h after induction, incorporation of [^{35}S]methionine into a culture harboring pPY2 was still 35% of the incorporation into a culture harboring YEp51-S1. The total RNA extracted from RNase III-induced cells exhibited an unchanged pattern of rRNA on ethidium bromide-stained gels. RNA blot analysis of actin, *LEU2*, and RNase III mRNA from *S. cerevisiae* exhibited no significant differences in the amounts of these mRNAs because of RNase III induction. In addition, at 12 h after induction of RNase III, incorporation of [^{32}P]phosphate and [^{35}S]methionine appeared to be affected to the same degree (total incorporation was about 35% when compared with that of a control culture). These results do not support the hypothesis that RNA, in general, is destabilized by expression of RNase III in *S. cerevisiae*. Instead, the effect seems to be on a specific target, presumably a cytoplasmic dsRNA moiety.

Resistance to RNase III induction. As described earlier, 12 to 15 h after induction of cultures harboring pPY2, variants resistant to RNase III induction were isolated at a frequency of 4×10^{-5} to 5×10^{-5}. We examined the ability of RNase III-resistant variants to produce toxin encoded by the M dsRNA. Two hundred independently isolated variant colonies were spotted onto a lawn of a toxin-sensitive strain. We found that 10% of these RNase III-resistant variants did not produce detectable amounts of toxin by this assay. All these killer-negative RNase III-resistant strains were found to lose M dsRNA concomitantly (Fig. 5B, lanes 1 to 3). In both killer-positive and killer-negative strains, the L dsRNA appeared to be intact (Fig. 5B, lanes 1 to 6). In a similar sample of induced cultures harboring YEp51-S1, no toxin-negative strains were isolated. As mentioned earlier, neither the stability nor the synthesis of dsRNA appeared to be affected by RNase III induction; thus, it is not clear how M dsRNA was cured in some RNase III-resistant variants. It is possible that RNase III has a subtle effect on stability or segregation of this dsRNA. Since it is known that cycloheximide (7), 5-fluorouracil (17), and yeast growth at high temperatures (26) can cure the cell of M dsRNA, it is also possible that M dsRNA was cured as a result of some metabolic changes caused by RNase III induction.

It was of interest to examine these RNase III-resistant variants further to determine whether the mutation occurred on the chromosome or on the plasmid. Southern blot analysis of K^+ and K^- strains (data not shown) revealed that both the plasmid and its 850-base-pair RNase III gene insertion appeared to be unaltered. Surprisingly, it was found that, by densitometric scanning of SDS-polyacrylamide-protein gels, similar amounts of RNase III protein were produced after induction of pPY2 in both the wild-type and variant strains (data not shown). The variants tested at different times after induction also exhibited the same level of RNase III activity as the wild type, as shown by two different assay systems described earlier (data not shown).

RNase III resistance due to a mutation in the RNase III gene. To determine whether the genetic change accompanying RNase III resistance was chromosomal or occurred within the plasmid pPY2, one of the K^+ RNase III-resistant variants was analyzed further. This variant was cured of its plasmid, and conversely the plasmid itself was isolated from the variant cells. When wild-type cells transformed with either pPY2 or the variant plasmid (designated SP1) were compared, only cells containing the original plasmid, pPY2, displayed the lethal phenotype. When the variant strain (designated BK3) harboring these same plasmids was checked, cells harboring pPY2 displayed the lethal phenotype, whereas cells harboring the variant plasmid were resistant to induction. These results indicate that RNase III resistance was due to the variant plasmid. Finally, the 850-base-pair RNase III-containing fragment from the variant plasmid was cloned into the original YEp51 vector. Cells harboring this plasmid did not exhibit the lethal phenotype, confirming that the genetic alteration leading to RNase III resistance had occurred within the 850-base-pair RNase III gene-containing insert.

The results described above indicate that the RNase III resistance mutation occurred in the structural gene of RNase III. The production of the altered RNase III appeared to be at the same level as that of the wild-type RNase III (Fig. 6). Furthermore, the mutated RNase was fully active when tested with dsRNA as a substrate. Therefore, the mutation in RNase III possibly changes the specificity of the enzyme in vivo in such a way that it does not recognize its lethal target anymore. In this regard it should be noted that RNase III specifically cleaves only certain single-stranded RNAs of *E. coli* in vivo (6). Alternatively, RNase III may require a yeast

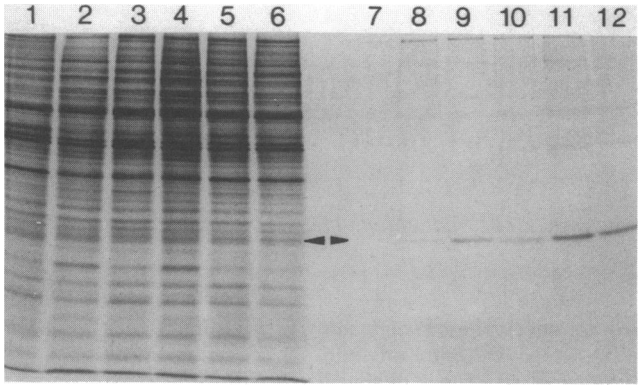

FIG. 6. Production of RNase III in yeast strains transformed with original and variant plasmids. Cell labeling, immunoprecipitation, and electrophoresis were as described in the legend to Fig. 1. Extracts of induced cultures of DMM1-15A::YEp51-S1 (lane 1), BK3::pPY2 (lane 2), BK3::SP1 (lane 3), DMM1-15A::pPY2 (lane 4), DMM1-15A::SP1 (lane 5), and an original RNase III-resistant variant, K^+2 (lane 6), were used. Lanes 7 to 12 contained immunoprecipitated samples of extracts shown in lanes 1 to 6, respectively. pPY2, original RNase III-containing plasmid; SP1, variant plasmid; DMM1-15A, wild-type strain; BK3, a resistant strain cured of its plasmid.

component for its activity in vivo. A mutation in RNase III blocking the interaction between these two components would result in loss of the lethal activity in vivo. Since the native RNase III enzyme has been shown to be a dimer of two homologous polypeptides (5), it is also possible that a mutation rendering the protein unable to form dimers would affect its activity in vivo. In this respect, it is interesting to examine whether the mutated RNase III gene from *S. cerevisiae* can complement a chromosomal RNase III mutation in *E. coli* (13).

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